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Abstract: Several types of liposomes were used to enrich Artemia nauplii in vitamin A, vitamin C and free methionine. In a first experiment, unilamellar liposomes formulated with krill phospholipid extract and retinyl palmitate demonstrated their capability to enhance the retinol content of Artemia nauplii. Furthermore, the increase in retinol was related to the amount of retinyl palmitate included in the liposomes as vitamin A source. These findings yield the possibility of using such vesicles to bioencapsulate simultaneously both vitamin A and essential fatty acids present in the krill phospholipid extract. A second enrichment was carried out with unilamellar liposomes composed of soybean phosphatidylcholine and loaded with sodium ascorbate as vitamin C source. Our results did not show that vitamin C content in the nauplii could be increased using unilamellar liposomes. This was most likely due to the degradation of the vitamin C during enrichment as well as the ascorbate leakage. Finally, a third experiment assessed enrichment in free methionine using liposomes of different lamellarity (unilamellar or multilamellar) and composed of either soybean phosphatidylcholine or dipalmitoyl phosphatidylcholine, both combined with cholesterol as a membrane stabilizer. Results indicated that multilamellar liposomes represent a useful tool to deliver methionine to Artemia nauplii. Enhanced protection given by their multiple bilayers in comparison to unilamellar liposomes could account for the higher ability displayed by multilamellar vesicles for free methionine bioencapsulation.

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15

## 16 Abstract

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 18 vitamin C and free methionine. In a first experiment, unilamellar liposomes formulated  
 19 with krill phospholipid extract and retinyl palmitate demonstrated their capability to  
 20 enhance the retinol content of *Artemia* nauplii. Furthermore, the increase in retinol was  
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 25 soybean phosphatidylcholine and loaded with sodium ascorbate as vitamin C source.

Our results did not show that vitamin C content in the nauplii could be increased using unilamellar liposomes. This was most likely due to the degradation of the vitamin C during enrichment as well as the ascorbate leakage. Finally, a third experiment assessed enrichment in free methionine using liposomes of different lamellarity (unilamellar or multilamellar) and composed of either soybean phosphatidylcholine or dipalmitoyl phosphatidylcholine, both combined with cholesterol as a membrane stabilizer. Results indicated that multilamellar liposomes represent a useful tool to deliver methionine to *Artemia* nauplii. Enhanced protection given by their multiple bilayers in comparison to unilamellar liposomes could account for the higher ability displayed by multilamellar vesicles for free methionine bioencapsulation.

Keywords: *Artemia* enrichment; liposomes; methionine; vitamin A; vitamin C

## 1. Introduction

Most of the studies on nutrition of marine fish larvae focus their attention on the importance of essential fatty acids (Izquierdo, 1996; Sargent et al., 1997). However, vitamins and free amino acids are also considered as essential nutrients, and therefore they have to be included in the diet for satisfying the requirements to maintain the different biological functions working correctly, and guarantee the normal growth (Rønnestad et al., 1999; Halver, 2002).

Vitamins are a heterogeneous group of compounds with a wide range of biological activities. Vitamins are classified according to their solubility, as either lipid or water soluble compounds (Gouillou-Coustans and Guillaume, 2001). Among liposoluble vitamins, vitamin A represents an essential nutrient for fish since they cannot synthesize this compound *de novo*. Vitamin A has been widely studied in fish because it is involved in vision and most of the fish reared species are visual feeders

(Hunter, 1981). Consequently, dietary deficiencies in vitamin A can alter normal function of vision in larvae, thereby reducing their predator ability. Moreover, dietary vitamin A deficiencies have been related to the occurrence of pseudoalbinism in flatfish such as halibut (*Hippoglossus hippoglossus*), turbot (*Psetta maxima*) and Japanese flounder (*Paralichthys olivaceus*). This syndrome is characterized by the lack of pigmentation in the body skin of the individual (Seikai et al., 1987; Estévez and Kanazawa, 1995). Furthermore, low vitamin A content in the live preys as *Artemia* can lead to an incomplete migration of the eye during flat fish metamorphosis (Saele et al., 2003). On the contrary, hypervitaminosis of vitamin A has been related to the backbone disease in Japanese flounder (Dedi et al., 1995, 1997), together with the jaw deformation (Haga et al., 2003).

In regard to hydrosoluble vitamins, vitamin C or ascorbic acid is a dietary essential nutrient for fish since these animals lack the enzyme gulonolactone oxidase required in its synthesis (Dabrowski, 1990). In addition to its essentiality, vitamin C also offers antioxidant (Hwang and Lin, 2002) and immunostimulant (Cuesta et al., 2002) properties, and protection against stress (Henrique et al., 2002). For these reasons, most diets used in aquaculture contain megadoses of vitamin C as a strategy to prevent diseases in cultured fish.

Amino acids are believed to be important dietary nutrients for early larval stages because it has been observed to be present in high amounts in fish eggs and in marine zooplankton, the food of the larvae (Rønnestad, 1992, Fyhn et al., 1993; Rønnestad et al., 1999). The main properties of free amino acids include their contribution to the formation of new tissues during body growth, their role as energy source supply (Rønnestad et al., 1999), and as effectors of chemical attraction (Kolkovski et al., 1997; Hara, 2006). Among essential amino acids, methionine can be considered to be a

76 limiting amino acid since its content in *Artemia* nauplii is only at trace or low levels  
77 (Helland et al., 2000).

78 Despite the fact that *Artemia* nauplii contain vitamin A, vitamin C and free  
79 amino acids (Merchie et al., 1997; Takeuchi et al., 1998; Helland et al., 2000),  
80 enrichment products normally include these compounds to enhance natural content of  
81 nauplii. However, inclusion of these compounds in the enrichment diet is somewhat  
82 difficult because of their different polarity. In the case of fish oil emulsions, one of the  
83 most habitual products employed in *Artemia* enrichment worldwide, lipid micelles  
84 formed when the product is dispersed in water possess a lipophilic core which impedes  
85 the incorporation of hydrosoluble molecules. In the case of vitamin C, this handicap has  
86 been overcome using lipophilic derivatives such as ascorbyl palmitate which is stable in  
87 the emulsion micelles (Merchie et al., 1995a, b). Finally, amino acids are water soluble  
88 compounds and, consequently, their inclusion in lipid emulsions seems to be difficult.

89 Liposomes represent a potential alternative for the enrichment of live preys in  
90 both hydrophilic and lipophilic nutrients (Hontoria et al., 1994). Simply stated,  
91 liposomes are lipid vesicles enclosing an aqueous space (New, 1990). According to this  
92 structure, liposomes can be formulated with water-soluble compounds dissolved in the  
93 aqueous space and lipophilic nutrients imbibed in the apolar moiety of phospholipid  
94 fatty acid chains. Previous studies have assessed the capacity of liposomes to deliver  
95 different compounds to live preys used in marine larviculture (Hontoria et al., 1994;  
96 Ozkizilcik and Chu, 1994; Touraki et al., 1995; Tonheim et al., 2000; Monroig et al.,  
97 2006).

98 The present study assesses several types of liposomes in the enrichment of  
99 *Artemia* nauplii in vitamin A, vitamin C and methionine as models for lipophilic  
100 vitamins, hydrophilic vitamins and amino acids, respectively.

## 101    **2. Materials and methods**

### 102    *2.1. Preparation of liposomes*

103            Liposomes prepared by means of three different methodologies were employed to  
 104    deliver vitamin A, vitamin C and methionine. Firstly, multilamellar vesicles (MLV)  
 105    were prepared by the method proposed by Bangham et al. (1965), but using filtered  
 106    seawater as the aqueous phase. Secondly, large unilamellar vesicles (LUV) were  
 107    obtained by extrusion (LUVext) of MLV suspensions using an extruder LiposoFast  
 108    equipped with 100 nm polycarbonate membranes (Avestin Inc., Ottawa, ON, Canada).  
 109    Finally, a second type of unilamellar liposomes was prepared by a detergent  
 110    solubilization methodology (LUVdet). In this latter typology, the aqueous phase  
 111    consisted of a saline solution (0.9% NaCl). More details about these methodologies are  
 112    found in Monroig et al. (2006).

113            Membranes of all three liposome typologies (MLV, LUVext and LUVdet) were  
 114    formulated with three different phospholipid sources purchased from Avanti Polar  
 115    Lipids (Alabaster, AL, USA): krill phospholipid extract (KPE), soybean  
 116    phosphatidylcholine (SPC), and dipalmitoyl phosphatidylcholine (DPPC). Lipid class  
 117    and fatty acid composition of phospholipids are presented in Table 1. Selection of the  
 118    membrane components and liposome typology was adopted according to the lipophilic  
 119    (retinyl palmitate) or hydrophilic (ascorbate and methionine) nature of the delivered  
 120    nutrient. Hence, liposomes chosen to enrich *Artemia* in vitamin A were composed of  
 121    KPE since this combination, although potentially leaky, would permit the simultaneous  
 122    enrichment in both essential fatty acids and vitamin A. Contrarily, vitamin C and  
 123    methionine were encapsulated in SPC and DPPC vesicles, which had demonstrated to  
 124    efficiently retain water-soluble substances when submitted to *Artemia* enrichment  
 125    conditions (Monroig et al., 2003). Occasionally, cholesterol (CHO) from Sigma

126 (Alcobendas, Spain) was included as a membrane stabilizer (New, 1990, p. 22).

127 Relevant features of the liposome suspensions used in this study are gathered in Table 2.

## 128 2.2. *Enrichment of Artemia nauplii in vitamin A*

129 Two types of unilamellar liposomes were used to enhance vitamin A of *Artemia*  
130 nauplii, together with the commercial emulsion Super Selco (Inve, Ghent, Belgium) as a  
131 reference product containing retinyl palmitate (Table 2). Both liposome suspensions  
132 were formulated with KPE and retinyl palmitate in a ratio of 100:1 (w/w) in LUVext  
133 (LUVext A), and 100:2 (w/w) in LUVdet (LUVdet A).

134 Commercial cysts (EG grade Inve, Ghent, Belgium) were incubated in aerated  
135 seawater at a temperature of  $28 \pm 1^\circ\text{C}$  with an illumination of 1500-2000 lux. The cyst  
136 incubation lasted for 23 h. During the following hour, the nauplii were separated from  
137 the cyst shells and stocked at approximate densities of 300 nauplii  $\text{mL}^{-1}$  in 1 l cylinder-  
138 conical enrichment tubes. At this point the nauplii were ready to begin the enrichment  
139 process ( $t_0$ ). Afterwards, the enrichment products were dispensed in a single dose  
140 yielding retinyl palmitate concentrations of 1.3, 5.0 and 10.0  $\text{mg l}^{-1}$  corresponding to  
141 Emulsion, LUVext A and LUVdet A, respectively. Similarly to the cyst incubation, the  
142 tubes were placed in a thermostated bath at a temperature of  $28 \pm 1^\circ\text{C}$  and illuminated  
143 between 1500-2000 lux. Aeration was applied in each enrichment tube with 0.5  
144 diameter section tubes, thereby yielding an approximate air flow of 1.0 litre per minute.

145 Samples of enriched nauplii were collected in a 100  $\mu\text{m}$  plankton mesh and  
146 gently washed with tap water, after 18 or 21 h of incubation in the enrichment medium.  
147 Part of the nauplii were kept in clean seawater at the same conditions of nauplii  
148 enrichment incubation stated above for 3 h more (24 h since the start of the incubation),  
149 and then sampled. This sample point reflects the nutritional value of nauplii after some



150 hours in the culture tank, and emulates food quality in normal hatchery procedures.  
 151 Enriched nauplii samples were immediately frozen until nutrient analyses performed.

### 152 2.3. *Enrichment of Artemia nauplii in vitamin C*

153 Newly obtained *Artemia* nauplii were incubated with four enrichment diets  
 154 (Table 2). In addition to the commercial emulsion Super Selco (Emulsion) that contains  
 155 ascorbyl palmitate as vitamin C source, *Artemia* nauplii were incubated with LUVdet  
 156 liposomes composed of SPC as a unique membrane component and prepared with  
 157 sodium ascorbate (Sigma, Alcobendas, Spain) dissolved in the aqueous phase at a  
 158 concentration of 100 mg ml<sup>-1</sup>. Liposomes were administered in a single dose at the  
 159 beginning of the incubation (t<sub>0</sub>) (LUVdet C 1) or in two separate doses at t<sub>0</sub> and t<sub>0</sub>+7 h  
 160 (LUVdet C 2). Finally, a fourth treatment consisted of sodium ascorbate directly  
 161 dissolved in the enrichment medium (Solution C). Both liposomes and solution  
 162 treatments yielded a sodium ascorbate concentration of 0.5 g l<sup>-1</sup>, whereas the ascorbyl  
 163 palmitate content of the commercial emulsion was not specified by the manufacturer.  
 164 Sampling was carried out as described in the vitamin A enrichment.

### 165 2.4. *Enrichment of Artemia nauplii in methionine*

166 Four liposome-based treatments were used to enhance the free methionine  
 167 content of *Artemia* nauplii (Table 2). LUVext formulated with SPC and CHO (4:1, w/w)  
 168 as membrane components and methionine dissolved in the aqueous phase (40 mg ml<sup>-1</sup>)  
 169 were administered in a single dose at t<sub>0</sub> (LUVext M 1) or in two separate doses at t<sub>0</sub> and  
 170 t<sub>0</sub>+8 h (LUVext M 2). A third enrichment product consisted of LUVext composed of  
 171 DPPC and CHO (4:1, w/w) and methionine dissolved in the aqueous phase (40 mg ml<sup>-1</sup>)  
 172 (LUVext M 3) administered in a single dose at the start of the nauplii incubation (t<sub>0</sub>).  
 173 Liposome treatments were completed with multilamellar vesicles (MLV M) with an  
 174 equal composition than LUVext M 1 and LUV M 2, and administered in a single dose at

175 the beginning of incubation. As performed in the vitamin C experiment, liposomes were  
176 compared with the efficiency displayed by methionine directly dissolved in the  
177 enrichment medium and dispensed at  $t_0$  (Solution M). In all five treatments,  
178 concentration of methionine in the enrichment medium was  $0.2 \text{ g l}^{-1}$  after administration  
179 of the total amount of product.

#### 180 *2.5. Determination of vitamin A*

181 Analysis of retinol was carried out following the method proposed by Takeuchi  
182 et al. (1998) with the following modifications. Total lipids were extracted from  
183 biological samples (around 10 mg DW) according to the method described by Folch et  
184 al. (1957), and then transferred to amber vials to avoid photodegradation. Lipid extracts  
185 were dissolved in a methanol:acetone solution (1:1. v/v) containing  $3.0 \text{ } \mu\text{g ml}^{-1}$  of  
186 retinyl acetate (Sigma, Alcobendas, Spain) used as an internal standard (Aust et al.,  
187 2001).

188 Lipid extracts (20  $\mu\text{l}$ ) were injected in a high performance liquid  
189 chromatography (HPLC) system (Thermo Liquid Chromatography, San Jose, CA,  
190 USA) equipped with a quaternary pump P4000 employed at isocratic conditions.  
191 Detection was performed using a UV-VIS UV6000LP detector set at a wavelength of  
192 325 nm. An oven Croco-Cil (Cluzeau, Sainte-Foy-la-Grande, France) was used to keep  
193 a C-18 Lichrospher (Merck, Darmstadt, Germany) at  $30^\circ\text{C}$  during the elution. The  
194 mobile phase consisted of 98% methanol containing 0.5% ammonium acetate (w/v) and  
195 chloroform 85:15 (v/v) which was pumped at a flow rate of  $1.5 \text{ ml min}^{-1}$

196 Peak identification was performed by comparing with commercial standards  
197 obtained from Sigma (Alcobendas, Spain) and quantification of each peak was carried  
198 out by comparing its relative area with that of the internal standard retinyl acetate.

199 ChromQuest Software (Thermo Quest, San Jose, CA, USA) was used in peak  
200 integration.

#### 201 *2.6. Determination of vitamin C*

202 Analyses of ascorbate (vitamin C) were performed following the method  
203 described by Shiau and Hsu (1994). A weighed portion of enriched nauplii (around 10  
204 mg DW) was suspended in 2 ml of ice cold 5% (w/v) metaphosphoric acid (Sigma,  
205 Alcobendas, Spain) and homogenized with a tissue disrupter (Ika Labor Technik, Staufen,  
206 Germany) for 30 s in an ice bath. Homogenates were centrifuged at 3000 g for 5 min,  
207 and supernatants were filtered through a 0.45 µm-pore-size glass fiber-nylon syringe  
208 filter (Whatman, Clifton, NJ, USA).

209 Ascorbate extracts (20 µl) were injected in the same HPLC system as described  
210 in the vitamin A analysis. In this case, mobile phase consisting of an aqueous solution  
211 of 0.05 M KH<sub>2</sub>PO<sub>4</sub> (pH 2.8) was pumped at a flow rate of 0.9 ml min<sup>-1</sup>. Elution was  
212 carried out at room temperature and the relative peak area was compared with ascorbic  
213 acid (Sigma, Alcobendas, Spain) as an external standard using a UV–VIS detector set at  
214 254 nm. ChromQuest Software (Thermo Quest, San Jose, CA, USA) was used in peak  
215 integration.

#### 216 *2.7. Determination of free methionine*

217 A modification of the method described by Koop et al. (1982) was used in the  
218 free amino acid determination. This method is based on a pre-column derivatization  
219 with phenylisothiocyanate (PITC, Sigma, Alcobendas, Spain). Samples of enriched  
220 nauplii (around 10 mg DW) were extracted in 1 ml of 6% (w/v) trichloroacetic acid  
221 (Panreac, Castellar del Vallés, Spain) using a tissue disrupter (Ika Labor Technik, Staufen,  
222 Germany). After maintaining homogenates for 24 h at 4°C, they were centrifuged

223 (10000 g) for 20 min, and supernatants were filtered through a 0.45  $\mu$ m-pore-size glass  
224 fiber-nylon syringe filter (Whatman, Clifton, NJ, USA).

225 Derivatization of free amino acids was accomplished after mixing 125  $\mu$ l of the  
226 filtrate and 25  $\mu$ l of 2 mM of norleucine used as an internal standard (Sigma,  
227 Alcobendas, Spain) with the following procedure. After desiccation of the mixture in a  
228 speed vacuum, 20  $\mu$ l of methanol:distilled water:trimethylamine (2:2:1, v/v/v) were  
229 added to facilitate complete desiccation, and again introduced in the speed vacuum.  
230 Finally, 15  $\mu$ l of freshly prepared derivatizing reagent composed of methanol:distilled  
231 water:trimethylamine:PITC (7:1:1:1, v/v/v/v) were added and incubated for 20 min at  
232 room temperature. After this period, derivatized samples were completely desiccated in  
233 the speed vacuum. At this point, samples were preserved at -20°C until HPLC injection.

234 Sample injection (20  $\mu$ l) was carried out after dissolving derivatized samples  
235 with 200  $\mu$ l of 5 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.4) containing 5% (v/v) acetonitrile. A C-18  
236 Lichrospher (Merck, Darmstadt, Germany) column maintained at 52°C during the  
237 elution was employed. Injected samples were eluted through a gradient mixture of two  
238 mobile phases: mobile phase A consisted of an aqueous solution of 70 mM sodium  
239 acetate (pH 6.55) containing 2.5% (v/v) acetonitrile; and mobile phase B was composed  
240 of acetonitrile:distilled water:methanol (45:40:15, v/v/v). All solvents were HPLC  
241 grade (Merck, Darmstadt, Germany). The flow rate of the mobile phase was 1 ml min<sup>-1</sup>,  
242 and followed the gradient program detailed in Table 3. The same UV-VIS detector as  
243 described above was employed at 254 nm. Peaks were identified by comparison with  
244 the amino acid commercial standard AA-S-18 (Sigma, Alcobendas, Spain), whereas  
245 quantification was obtained by comparing its relative area with that of the internal  
246 standard norleucine. ChromQuest Software (Thermo Quest, San Jose, CA, USA) was  
247 used in peak integration.

## 248 2.8. Statistical analyses

249 Analytical data are expressed as means  $\pm$  standard deviations. Homogeneity of  
 250 variances was checked by Barlett's test. In cases of homoscedasticity, differences in the  
 251 nutrient content among treatments in each sampling point were analyzed by one-way  
 252 analysis of variance (ANOVA) followed, where appropriate, by Tukey's multiple  
 253 comparison test (Sokal and Rohlf, 1981). If heterogeneity of variances existed, robust  
 254 tests were applied. Welch's test was used to check differences between treatments and  
 255 Game-Howell's test to establish differences among groups. Comparisons of the means  
 256 with  $P$  values equal or less than 0.05 were considered significantly different. All the  
 257 statistical analyses were carried out using the SPSS statistical package (SPSS Inc.,  
 258 Chicago, IL, USA).

## 259 3. Results

### 260 3.1. Enrichment of *Artemia nauplii* in vitamin A

261 Fig. 1 shows the retinol content of nauplii enriched with two different liposome  
 262 preparations and the commercial emulsion. A relationship between the amount of  
 263 retinyl palmitate present in the enrichment diet and the retinol measured in the treated  
 264 nauplii can be observed. Thus, nauplii enriched with LUVdet A displayed higher retinol  
 265 levels than the other two treatments in all three sampling points ( $P \leq 0.05$ ). At the same  
 266 time, LUVext A nauplii show higher retinol concentration than nauplii enriched with  
 267 the commercial emulsion after 18 and 21 h of incubation, although no differences can  
 268 be seen at the 24 h sampling point ( $P \leq 0.05$ ). It is noteworthy to mention that nauplii  
 269 enriched with the emulsion, that includes retinyl palmitate in its composition, exhibit a  
 270 retinol contents below the detection threshold. In regards to retinol incorporation  
 271 throughout enrichment, both liposome-based treatments did not exhibit important

changes from 18 to 21 h. Nonetheless, the subsequent starvation period (24 h sampling point) produced a drop in the retinol content of liposome enriched nauplii to the extent that retinol from LUVext A nauplii was below detection threshold at the 24 h sampling point (Fig. 1).

### 3.2. *Enrichment of Artemia nauplii in vitamin C*

Ascorbate analyses from nauplii enriched with the different products are shown in Fig. 2. In general, nauplii enriched with the commercial emulsion contained lower ascorbate levels in comparison to the other treatments. This difference became significant ( $P \leq 0.05$ ) at 18 h of incubation. Another remarkable result is the lack of significant differences at any sampling point ( $P \leq 0.05$ ) between the ascorbate content of nauplii enriched with liposomes administered in a single dose (LUVdet C 1) and two doses (LUVdet C 2). Nauplii incubated with dissolved ascorbate (Solution C) showed not only statistically equivalent levels than the other treatments, but also their ascorbate content was even higher ( $P \leq 0.05$ ) than the other's after the starvation period. Concerning the temporal incorporation, ascorbate levels from nauplii did not undergo conclusive patterns, except for the increase observed during the starvation period in nauplii treated with dissolved ascorbate (Fig. 2).

### 3.3. *Enrichment of Artemia nauplii in free methionine*

Levels of free methionine measured in *Artemia* nauplii incubated with different enrichment products are represented in Fig. 3. After 18 h of incubation, nauplii enriched with MLV liposomes (MLV M) registered significantly higher contents of free methionine in comparison to the other treatments ( $P \leq 0.05$ ). Such a difference was maintained for up to 21 h, although LUVext M 2 (2 doses) did not differ from MLV nauplii ( $P \leq 0.05$ ) at that sampling point. Although this tendency is also observed at 24 h,

296 free methionine of nauplii enriched with MLV liposomes only showed significant  
297 higher contents if compared to LUVext M 1 and Solution M nauplii. As seen in the  
298 retinol and ascorbate experiments, temporal evolution of the free methionine levels of  
299 nauplii showed minor changes from 18 to 21 h, except for LUVext M 1 nauplii, which  
300 seemed to exhibit an increase during this period. On the contrary, the starvation period  
301 was accompanied by a general decrease in the free methionine visible in all five  
302 treatments.

#### 303 **4. Discussion**

304 The ability of liposomes to deliver both hydrophilic and lipophilic substances  
305 has been employed to improve the nutritional value of live preys used for feeding  
306 marine fish larvae (Ozkizilcik and Chu, 1994; McEvoy et al., 1996; Tonheim et al.,  
307 2000). With the exception of the pioneer study undertaken by Hontoria et al. (1997) on  
308 vitamin encapsulation with liposomes, the present study represents the first report of  
309 vitamin A bioencapsulation in live preys by means of these lipid vesicles. Some  
310 nutritional studies on flat fish larvae have assessed the efficiency of several compounds  
311 with vitamin A activity (i.e., retinol, retinyl esters, etc.) included in oil emulsions and  
312 bioencapsulated in *Artemia* nauplii (Dedi et al., 1995; Estévez and Kanazawa, 1995;  
313 Takeuchi et al., 1998; Haga et al., 2004). Results obtained in the present study illustrate  
314 the efficiency of liposomes formulated with krill phospholipids and retinyl palmitate to  
315 enhance the retinol content in *Artemia* nauplii. Additionally, the amount of retinol in the  
316 nauplii can be directly related to the retinyl ester included in the liposome membrane,  
317 thereby pointing at a metabolic transformation by naupliar enzymatic system as  
318 previously described by Takeuchi et al. (1998). Retinyl palmitate incorporated in  
319 liposomes would be hydrolyzed to free retinol before its absorption in the intestinal cells  
320 (Furr and McGrane, 2003). Metabolic activity of nauplii may also explain the decrease

321 in the retinol levels observed throughout the starvation period in liposome enriched  
322 nauplii, probably due to a reesterification of retinol (Furr and McGrane, 2003) within  
323 digestive tract cells. A shocking result is the low retinol level obtained in nauplii  
324 incubated with the commercial emulsion despite containing retinyl palmitate. This poor  
325 efficiency of the commercial emulsion may, in part, be due to a partial degradation  
326 during the incubation of the enrichment product under aggressive conditions of light,  
327 oxygen and temperature (Woollard and Indyk, 2003). Indeed, Moren et al. (2005) have  
328 recently proposed this degradation mechanism to explain why no vitamin A was  
329 detected when another enrichment product (DC-DHA Selco) was submitted to  
330 simulated enrichment conditions during 12 h. Despite the fact that liposomes could  
331 undergo the same process, the higher amount of retinyl palmitate present in liposomes  
332 in comparison to the commercial emulsion would preserve enough quantity to be  
333 bioencapsulated in the *Artemia* nauplii before degradation.

334       Regarding the vitamin C enrichment, our results revealed that enhancement in  
335 the ascorbate content of *Artemia* nauplii was more efficient when ascorbate was  
336 encapsulated in liposomes or simply dissolved in the water in comparison to the oil  
337 emulsion formulated with ascorbyl palmitate. The importance of such a result is  
338 difficult to evaluate since the amount of vitamin C available in all treatments was not  
339 the same. Whereas nauplii treated with liposomes and the aqueous solution were  
340 incubated at the same initial ascorbate concentration in the medium ( $0.5 \text{ g l}^{-1}$ ), the  
341 amount of vitamin C available for nauplii enriched with the commercial emulsion  
342 depends on the concentration of ascorbyl palmitate present in the product (not specified  
343 by the manufacturer), along with the efficiency of the nauplii to hydrolyze the ester  
344 derivative to free ascorbate (Merchie et al., 1995a). However, results do not prove the  
345 presumable advantage of using liposomes instead of the aqueous solution, since



346 differences between liposomes and the aqueous solution did not exist. Firstly, the low  
347 efficiency of liposomes to bioencapsulate ascorbate in *Artemia* can be partly explained  
348 through the susceptibility of ascorbate to degrade (Halver, 2002) as occurred in vitamin  
349 A. Preservation of vitamin C by means of other antioxidant compounds such as vitamin  
350 E included in the membrane (Urano et al., 1987; Fukuzawa et al., 1993a, b) or the  
351 utilization of more resistant derivatives (Schüep et al., 1989; Shiao and Hsu, 1994;  
352 Yoshitomi, 2004) would improve the efficiency of liposomes as an ascorbate carrier to  
353 *Artemia* nauplii. Secondly, the turbulent regime of enrichment would produce the  
354 leakage of water-soluble compounds encapsulated in liposomes. This phenomenon,  
355 which is expected to affect other hydrosoluble substances such as methionine, is  
356 discussed later on.

357         Results from the methionine enrichment indicate the possibility of using  
358 liposomes to enhance the free amino acid pool in *Artemia* nauplii. Nauplii enriched with  
359 multilamellar liposomes (MLV) presented higher levels of free methionine than  
360 unilamellar liposomes (LUV), despite the fact that LUV are considered to have the most  
361 efficient typology to maximize encapsulation of water-soluble compounds because of  
362 the high internal volume:membrane lipid ratio (New, 1990, p. 28). Nevertheless, under  
363 high turbulence conditions of enrichment procedures, MLV formulations seem to be  
364 more suitable than LUV possibly due to their protective multiple bilayer structure  
365 (between 5 and 20 layers). Therefore, methionine dissolved in the more internal  
366 intermembrane spaces of MLV would remain encapsulated, whereas the turbulence  
367 during incubation would cause the leakage of water-soluble compounds from the  
368 aqueous compartments located between the more outer membranes, as would also occur  
369 in the unique aqueous space of LUV. This low performance of LUV could neither be  
370 improved through the dosage of liposomes in two separate doses, despite the fact that

371 this strategy would increase the time during which vesicles are still loaded with the  
372 water-soluble nutrient (Monroig et al., 2003). Consequently, bioencapsulation of  
373 methionine loaded particles (MLV liposomes) would be favoured in comparison to  
374 methionine simply dissolved in the enrichment medium (case of original methionine  
375 solution and empty unilamellar liposomes) since filtering appendages of nauplii are  
376 adapted to retain discrete particles of a certain size present in the water column.

377         The direct incorporation of methionine through ingestion of enrichment medium  
378 by nauplii should also be considered, as *Artemia* nauplii drink medium for  
379 osmoregulatory balance (Navarro et al., 1993). Considering the content of free  
380 methionine from newly hatched nauplii ( $88.4 \mu\text{g g DW}^{-1}$ ) as the basal level, free  
381 methionine measured in nauplii enriched with the methionine solution (Solution M)  
382 (between 300 and  $400 \mu\text{g g DW}^{-1}$ ) possibly indicate a net incorporation of the amino  
383 acid through ingestion of the medium. The existence of this direct pathway was  
384 demonstrated in *Artemia* enrichments with radioactive methionine (Tonheim et al.,  
385 2000). In regards to temporal incorporation, analyses did not indicate an important  
386 increase in the free methionine content during the period between 18 and 21 h. Despite  
387 the supply of methionine during this period, the free methionine already  
388 bioencapsulated in the nauplii could not be detected since nauplii can assimilate it into  
389 the protein structure, transform it into other amino acids (cysteine or taurine), and  
390 oxidize it to sulphate products (Tonheim et al., 2000). Moreover, these mechanisms  
391 could still occur during the starvation period (from 21 to 24 h), thus explaining the drop  
392 of free methionine levels in all treatments. Indeed, total absorbed methionine was  
393 estimated to be two fold compared to that present in a free state (Tonheim et al., 2000).

394         In light of these results, unilamellar vesicles cannot be considered the best  
395 alternative to encapsulate hydrophilic nutrients in liposomes employed in *Artemia*

enrichments, contrarily to the results obtained from another study (Monroig et al., 2003) where LUV suspensions retained a fluorescent marker during a longer period than MLV liposomes. This apparent discrepancy can be related to several reasons. Firstly, MLV suspensions tested by Monroig et al. (2003) were formulated with krill phospholipids which would form leaky vesicles due to the unsaturation degree of their fatty acid chains (Alberts et al., 1996, p.512-513). On the contrary, formulation of multilamellar liposomes with purified soybean phosphatidylcholine in the present study would improve the retention of water-soluble substances included in these vesicles. Secondly, differences in the hydrodynamic conditions set in both studies could also explain the different behaviour of multilamellar liposomes. Incubation of nauplii in the present study was carried out in 1 l tubes with an aeration flow around 1.0 lpm, whereas we previously used smaller tubes (200 ml) where aeration was reduced to simulate normal turbulence applied in standard 1 l enrichment tubes (Monroig et al., 2003). While attempting to standardize turbulence in the 200 ml tubes, the degree of agitation in the enrichment medium in this study could have resulted to be higher than the previous study by Monroig et al. (2003). Finally, differences between the solubility of the fluorescent marker carboxyfluorescein used in our preliminary experiment (Monroig et al., 2003) and the water-soluble substances used here, such as methionine, could affect their leakage rate and, consequently, make results from both studies not fully comparable.

In summary, liposomes can be presented as an efficient system for the enrichment of *Artemia* nauplii in retinol. Furthermore, retinol incorporation was related to the amount of retinyl palmitate included in the liposome formulation. These findings demonstrate the potential of these vesicles to bioencapsulate simultaneously both vitamin A and essential fatty acids present in the krill phospholipid extract. Regarding

421 water-soluble nutrients, the present study could not demonstrate the efficiency of  
 422 unilamellar liposomes to increase the vitamin C content of nauplii probably due to  
 423 degradation during the enrichment process. The use of more stable derivatives, together  
 424 with the combination with other antioxidant compounds could improve liposome  
 425 capacity for vitamin C enrichment. In addition, leakage of vitamin C could be another  
 426 factor limiting the liposome efficiency. As shown in the methionine enrichment,  
 427 multilamellar liposomes could be a good candidate to deliver hydrosoluble compounds  
 428 to nauplii. Enhanced protection given by their multiple bilayers could account for a high  
 429 ability of water-soluble retention inside the liposomes. In these circumstances, nauplii  
 430 would incorporate particulate liposomes more efficiently than dissolved molecules by  
 431 means of their adapted filtering-natatory appendages.

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## 578 Tables

579 Table 1. Selected fatty acid (percent of total fatty acids) and lipid class composition  
 580 (percent of total lipids) of phospholipid sources used in the liposome formulation.

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	KPE	SPC	DPPC
<i>Fatty acid</i>			
16:0	25.5	15.1	100.0
18:0	1.0	3.0	0.0
18:1n-9	5.4	13.6	0.0
18:2n-6	1.8	60.6	0.0
18:3n-3	1.4	3.6	0.0
20:4n-6	0.7	0.0	0.0
20:5n-3	29.7	0.0	0.0
22:5n-3	0.8	0.0	0.0
22:6n-3	18.4	0.0	0.0
Saturates	28.6	18.7	100.0
Monounsaturates	13.2	15.8	0.0
Polyunsaturates	55.8	64.5	0.0
Total n-3	52.5	3.8	0.0
Total n-6	3.2	60.7	0.0
HUFA n-3	49.3	0.2	0.0
HUFA n-6	1.4	0.1	0.0
DHA/EPA	0.6	0.0	0.0
<i>Phospholipid class</i>			
PC	67.0	95.0	100.0
PE	9.0	0.0	0.0
Other	24.0	5.0	0.0

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581 HUFA n-3:  $\geq 20:3n-3$ ; HUFA n-6:  $\geq 20:2n-6$ ; DHA/EPA: docosahexaenoic to

582 eicosapentaenoic fatty acid ratio; PC: phosphatidylcholine; PE:

583 phosphatidylethanolamine.

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586

Table 2. Enrichment products assessed in the *Artemia* enrichment in vitamin A, vitamin C and methionine.

<i>Delivered nutrient</i>	<i>Enrichment products</i>	<i>Lamellarity (liposomes)</i>	<i>Preparation method (liposomes)</i>	<i>Dosage</i>	<i>Membrane composition (w/w)</i>
<i>Vitamin A</i>	Emulsion	-	-	1	-
	LUVext A	Unilamellar	Extrusion	1	KPE: vitA (100:1)
	LUVdet A	Unilamellar	Detergent solubilization	1	KPE: vitA (100:2)
<i>Vitamin C</i>	Emulsion	-	-	1	-
	LUVdet C 1	Unilamellar	Detergent solubilization	1	SPC (100%)
	LUVdet C 2	Unilamellar	Detergent solubilization	2	SPC (100%)
	Solution C	-	-	1	-
<i>Methionine</i>	LUVext M 1	Unilamellar	Extrusion	1	SPC: CHO (4:1)
	LUVext M 2	Unilamellar	Extrusion	2	SPC: CHO (4:1)
	LUVext M 3	Unilamellar	Extrusion	1	DPPC: CHO (4:1)
	MLV M	Multilamellar	Simple lipid hydration	1	SPC: CHO (4:1)
	Solution M	-	-	1	-

LUVdet: large unilamellar vesicles prepared by the detergent solubilization

methodology; LUVext: large unilamellar vesicles prepared by extrusion through

polycarbonate membranes; MLV: multilamellar vesicles; KPE: krill phospholipid

extract; SPC: soybean phosphatidylcholine; DPPC: dipalmitoyl phosphatidylcholine;

CHO: cholesterol; vitA: retinyl palmitate.

Table 3. Gradient elution program followed in the analysis of free amino acids.

<u>Time (min)</u>	<u>% phase A</u>	<u>% phase B</u>
0.0	100.0	0.0
13.5	97.0	3.0
16.5	97.0	3.0
19.0	96.5	3.5
21.0	95.5	4.5
24.0	94.0	6.0
25.0	93.0	7.0
26.5	92.0	8.0
28.5	91.0	9.0
30.0	91.0	9.0
50.0	66.0	34.0
65.0	30.0	70.0

596 Mobile phase A, aqueous solution of 70 mM sodium acetate (pH 6.55) containing 2.5%  
597 (v/v) acetonitrile; Mobile phase B, solution of acetonitrile:distilled water:methanol  
598 (45:40:15, v/v/v).

599

600

601 Figure legends

602 Figure 1. Contents of retinol ( $\mu\text{g}$  per gram of dry weight) of *Artemia* nauplii enriched  
603 with different products. Samples of 24 h belong to nauplii kept during 3 h in clean  
604 seawater after 21 h of incubation with the enrichment product. Data are means of three  
605 replicates. Error bars represent standard deviations. Treatments sharing the same  
606 superscript letter in a sampling point are not significantly different from each other  
607 ( $P \leq 0.05$ ).

608 Figure 2. Contents of ascorbate (mg per gram of dry weight) of *Artemia* nauplii  
609 enriched with different products. Samples of 24 h belong to nauplii kept during 3 h in  
610 clean seawater after 21 h of incubation with the enrichment product. Data are means of  
611 three replicates. Error bars represent standard deviations. Treatments sharing the same  
612 superscript letter in a sampling point are not significantly different from each other  
613 ( $P \leq 0.05$ ).

614 Figure 3. Contents of free methionine ( $\mu\text{g}$  per gram of dry weight) of *Artemia* nauplii  
615 enriched with different products. Samples of 24 h belong to nauplii kept during 3 h in  
616 clean seawater after 21 h of incubation with the enrichment product. Data are means of  
617 three replicates. Error bars represent standard deviations. Treatments sharing the same  
618 superscript letter in a sampling point are not significantly different from each other  
619 ( $P \leq 0.05$ ).

620

Figure 1

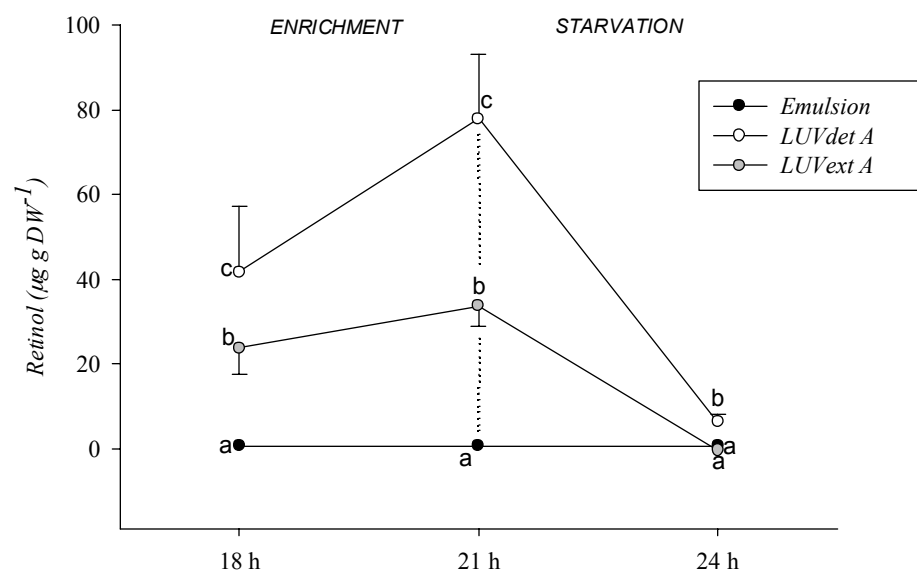


Figure 2

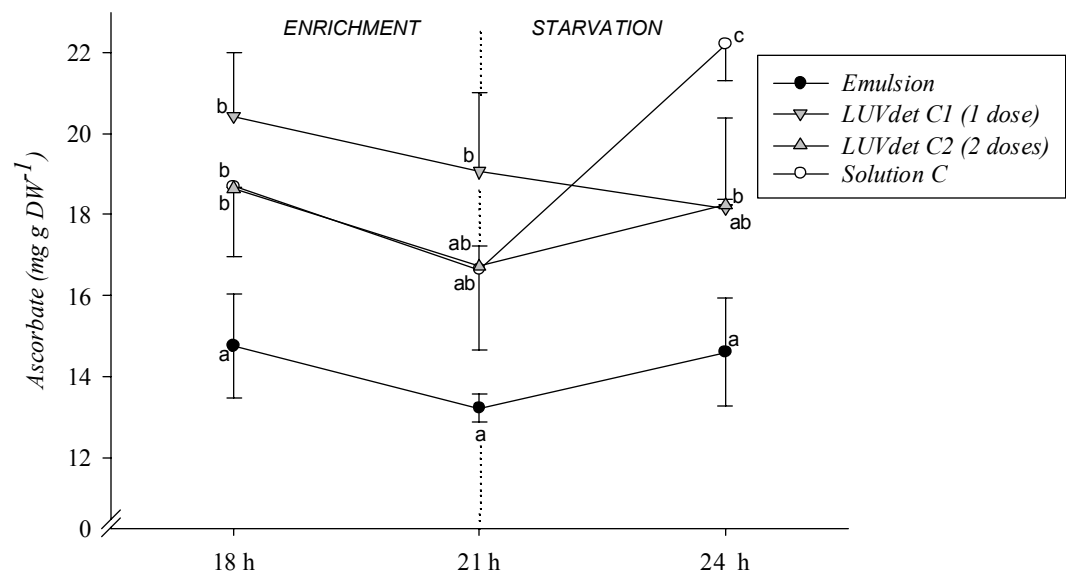


Figure 3

